

Characterization of a Messenger RNA Polynucleotide Vaccine Vector¹

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Abstract

We have constructed mRNA transcripts encoding luciferase and human carcinoembryonic antigen (CEA) which are capped, polyadenylated, and stabilized by human β -globin 5' and 3' untranslated regions. The mRNA construct encoding human CEA directed CEA expression in mouse fibroblasts *in vitro* following liposome-mediated transfection. The luciferase encoding mRNA transcripts mediated luciferase expression *in vivo* following i.m. injection. Based on the demonstration of protein expression *in vitro* and *in vivo*, the feasibility of using such a vector as a tumor vaccine was examined. In this pilot study, seven mice received 50 μ g mRNA transcripts encoding CEA twice weekly for 5 weeks by i.m. injection followed by challenge with syngeneic, CEA-expressing tumor cells. This dose and schedule "primed" an immune response to CEA. Five of seven mRNA-immunized mice demonstrated anti-CEA antibody 3 weeks after tumor challenge whereas control mice had no evidence of antibody response. This strategy might be particularly useful to induce an immune response to a proto-oncogene product or growth factor which poses a risk of inducing malignant transformation consequent to prolonged protein expression.

Introduction

The technique of direct i.m. injection of plasmid DNA encoding specific antigens has been used as a means to achieve highly specific immunization. This polynucleotide vaccine strategy has elicited humoral and cellular immune responses to a variety of infectious agents including influenza, hepatitis B, HIV, and others (1-3). The rationale for polynucleotide immunization derives from the following advantages: (a) it elicits both humoral and cell-mediated immune responses using a nonreplicating vector without adjuvants; (b) intracellular synthesis of the antigen favors MHC class I peptide display considered pivotal to the generation of cytolytic T cells (4); (c) gene expression in skeletal muscle following plasmid DNA injection has been detected for up to 19 months after injection favoring long-lived immunity (5, 6, 7); and (d) large quantities of purified DNA for vaccination can be prepared and standardized with relative ease compared to protein purification techniques. To examine the ability of polynucleotide immunization to achieve specific antitumor immunity, we have constructed a plasmid DNA encoding the full-length cDNA for human CEA³ under transcriptional regulatory control of the cytomegalovirus early promoter/enhancer (8). This plasmid can function as a polynucleotide vaccine to elicit CEA-specific humoral and cellular immune responses as well as protection against syngeneic, CEA-expressing colon carcinoma cells (8-10). These effects were comparable to the immune response and immunoprotection achieved with a recombinant vaccinia virus encoding CEA (8).

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³ The abbreviations used are: CEA, carcinoembryonic antigen; ORF, open reading frame.

In considering the application of this technology to additional tumor-associated antigens, it is noteworthy that many recently cloned tumor-associated antigens consist of the protein products of proto-oncogenes such as mutated *ras* or nonmutated proto-oncogenes such as *Her2/neu* (11-13). Prolonged cellular expression of mutated *ras* or overexpression of *Her2/neu* have been associated with malignant transformation (14-16). Thus, use of plasmid DNA encoding such proto-oncogenes for polynucleotide immunization would pose the risk of malignant transformation of host cells consequent to long-term proto-oncogene expression. As a strategy to circumvent this theoretical risk and facilitate the use of oncogene-derived tumor-associated antigens, we have considered the use of mRNA as the polynucleotide vehicle for immunization. The use of mRNA for gene therapy applications was first described by Malone *et al.* (17) in the context of liposome-mediated transduction. The ability of i.m. injection of naked mRNA to elicit gene expression was demonstrated by Wolff *et al.* (18) in their original description of *in vivo* transduction of muscle by direct i.m. injection of polynucleotides mRNA or DNA. Subsequently, we and others have demonstrated the utility of mRNA transcripts for transducing tumor cells *in vitro* and *in vivo* (19, 20). Studies of mRNA-mediated transfection both *in vitro* and by i.m. injection *in vivo* have demonstrated maximum reporter gene expression within 12-18 h with no expression detectable 72 h postinjection (18, 19). Thus, mRNA mediated transfection produces "self-limited" gene expression presumably related to intracellular mRNA enzymatic degradation. Furthermore, mRNA cannot integrate into the host genome because host cells lack endogenous reverse transcriptase required to convert mRNA to DNA. Thus, polynucleotide immunization with mRNA transcripts encoding oncogenic sequences would produce self-limited expression of the immunogen without potential for malignant transformation of host cells. In this report, we examine the feasibility of this strategy utilizing mRNA transcripts encoding human CEA including *in vitro* characterization of the vector and preliminary data regarding *in vivo* expression and immune response.

Materials and Methods

Derivation of Human CEA-encoding mRNA Transcripts. Translation-competent mRNA transcripts encoding human CEA were produced by *in vitro* synthesis using an SP6 DNA-directed RNA transcription system utilizing standard methods (21). The DNA template for the *in vitro* transcription reaction was derived by cloning of the human CEA cDNA ORF into the *Bgl*III site of the SP6 vector, SP64T (21). For this construction, the human CEA cDNA was mobilized from the plasmid pGT6 (8) by digestion with the restriction endonuclease *Sma*I. The purified 2.4-kilobase DNA fragment was then cloned into the pSP64T vector, which had been linearized by *Bgl*III and termini blunted by Kenow fill-in reaction. The SP64T vector contains a unique *Bgl*III site flanked by 5' and 3' untranslated regions derived from the human β -globin mRNA transcript. The recombinant SP64T-CEA construct would thus allow the synthesis of mRNA transcripts consisting of the human CEA ORF flanked by the human β -globin 5' and 3' untranslated regions. These regions have been shown to confer stability on other heterologous mRNA transcripts (17). For *in vitro* transcript synthesis, the plasmid SP64T-CEA was linearized 3' to the terminus of the synthetic poly(A) site and the linearized plasmid was purified by standard techniques. Capped, polyadenylated CEA

mRNA transcripts were then derived by *in vitro* synthesis with this template utilizing the Megascript (Ambion, Austin, TX) kit under conditions recommended by the manufacturer. After the *in vitro* synthesis reactions, the linearized DNA template was removed by digestion with DNase and the mRNA transcripts were purified by phenol/chloroform extraction followed by ethanol precipitation (17). The synthesized transcripts were size fractionated by agarose gel electrophoresis to determine size and purity. As control, mRNA transcripts were also derived which encoded the firefly luciferase reporter gene. These were synthesized from the plasmid pGT28, as described previously (19).

In Vitro Functional Validation of mRNA Transcripts Encoding Human CEA. The functional integrity of the CEA mRNA transcripts was validated based on their ability to direct synthesis of CEA protein in heterologous cells *in vitro*. WI62 cells, mouse fibroblasts lacking CEA expression, were transfected with the CEA mRNA using a liposome vector. The WI62 cells were obtained from the American Type Culture Collection and propagated in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were seeded into 6-well plates at 2×10^5 cells/well with each well containing a flame-sterilized glass coverslip. When the cells reached approximately 50% confluency on the coverslips, liposome-mRNA complexes were prepared by combining 2.5 µg CEA mRNA with 5 µg commercial cationic liposome DOTAP (Boehringer Mannheim, Mannheim, Germany) and incubating for 30 min at room temperature as described (17, 19, 20). The mRNA-liposome complexes were incubated with target cells in serum-free DMEM for 4 h followed by an additional 12-h incubation in DMEM-10% FCS prior to analysis of gene expression. Control cells were transduced in a similar manner with mRNA transcripts encoding the firefly luciferase reporter gene (19) or plasmid DNA-encoding human CEA cDNA, pGT37 (10), as described previously. In the case of plasmid DNA transduction, cells were incubated 24 h posttransduction prior to analysis of gene expression. The transduced cells were analyzed for CEA expression by immunostaining with the human CEA-specific mAb antibody COL-1 (22) using a streptavidin-peroxidase method. Bound antibody was detected with a biotinylated anti-mouse IgG secondary antibody and streptavidin-peroxidase complex using diaminobenzidine tetrahydrochloride as the substrate to yield brown staining. Counterstaining was performed with hematoxylin. Cell surface expression of CEA was evaluated by RIA of radiolabeled COL-1 binding. The cpm of radiolabeled COL-1 bound was converted to molecules of COL-1 bound per cell based on the specific activity as described previously (10).

Intramuscular Injection of Plasmid DNA and mRNA Transcripts. Six- to 8-week old female C57BL/6 mice (Charles River Laboratories, Raleigh, NC) were anesthetized with ketamine and xylazine by i.p. injection. Tongues were pulled out of the mouth gently with forceps to allow a 28-gauge needle to penetrate the bulk of the tongue muscle. All polynucleotide injections consisted of 50 µg plasmid DNA or mRNA transcripts in 50 µl RNase-free sterile saline. Preliminary studies by our laboratory and others (8–10, 23) have demonstrated that tongue injections of this volume do not impair the ability of animals to eat or drink following recovery from anesthesia. The tongue was selected to allow direct visualization of the striated muscle without necessitating a surgical procedure.

In Vivo Verification of mRNA Transcript Expression in Muscle. The relative magnitude and time course of heterologous protein expression following i.m. delivery of mRNA and plasmid DNA was analyzed using the firefly luciferase reporter gene. Groups of five mice received a single 50-µg dose of the luciferase encoding vectors, pGT28Luc mRNA or pCLuc4 DNA (19), by i.m. injection. Luciferase activity from a 10-µl aliquot of muscle extract was measured at various time points following injection. A positive result was defined as exceeding 2 SD above the mean for 10 control muscles injected with saline and was >700 light units.

Immune Response. To characterize the ability of mRNA transcripts encoding CEA to prime a CEA-specific immune response, seven mice received 50 µg CEA mRNA twice weekly for 5 weeks followed by *in vivo* CEA challenge 1 week later via injection of 2×10^5 syngeneic MC38-CEA-2 cells (24) as described previously (9, 10). Seven naive mice tumor challenged on the same day served as controls. These tumor cells are a mouse colonic adenocarcinoma line which expresses human CEA through retroviral transfection. Three weeks after tumor challenge, sera were obtained for evaluation of CEA-specific antibody response.

Antibody Assay. Anti-human CEA antibody was quantitated using a double antigen immunoradiometric assay as described previously (8–10, 25). Briefly, 6.4-mm polystyrene beads (Precision Plastic Ball, Chicago, IL) were

coated with purified human CEA (2 µg/bead) in PBS, washed three times with PBS containing 1% BSA, and stored in wash buffer at 4°C until use. Twenty µl mouse sera (normal control or postimmunization) were diluted to 100 µl with PBS and incubated with a single coated bead (in duplicate) for 2 h on a laboratory oscillator at room temperature, washed with PBS, and incubated with 100 µl 125 I-labeled human CEA (approximately 10^6 cpm/µg) at 2 µg/ml for 1 h, rewashed with PBS, and counted on a Micromedic automatic gamma counter. Background nonspecific binding of approximately 1% of the available 125 I-labeled CEA was subtracted from cpm bound and the ng of CEA bound to the bead per ml of sera was calculated from the known specific activity of the 125 I-labeled CEA. A positive response (>20 ng/ml) has been defined as exceeding 2 SD above the mean value of 10 normal mouse sera.

Results

To accomplish synthesis of functional mRNA transcripts encoding human CEA, a plasmid DNA vector containing the human CEA cDNA was constructed as illustrated in Fig. 1A. Linearization of the resulting plasmid, SP64T-CEA, formed the template for derivation of CEA mRNA transcripts. Synthesis of the transcripts was accomplished by an SP6 polymerase *in vitro* synthesis reaction using standard methods (21). The incorporation of modified guanil residues in the *in vitro* transcription reaction at a defined ratio allowed the recovery of mRNA transcripts capped at their 5' terminus. This maneuver has also been shown to be crucial to the translational efficacy of transcripts synthesized *in vitro* (17). After synthesis, the transcripts were analyzed by agarose gel electrophoresis (Fig. 1B). In

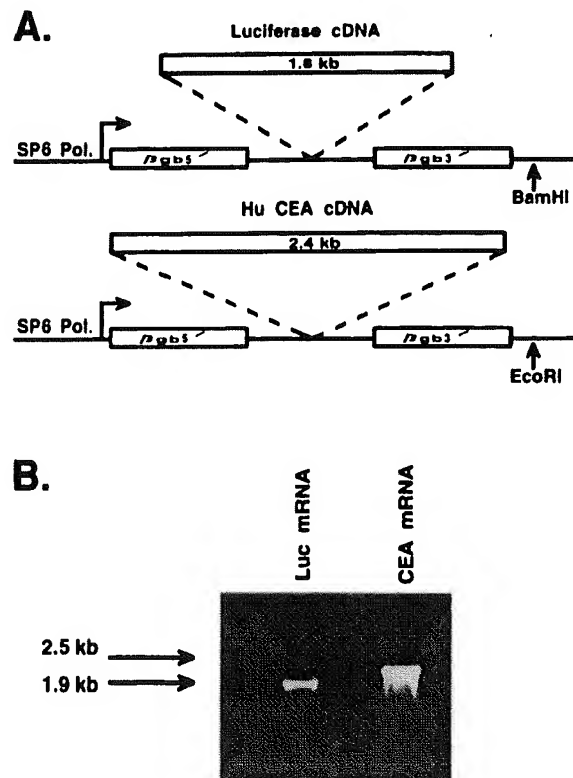


Fig. 1. Derivation of mRNA transcripts encoding firefly luciferase and human CEA. A, partial plasmid DNA maps of the SP6 plasmids used for the *in vitro* syntheses of mRNA transcripts. The plasmid SP64T contains sequences encoding the 5' and 3' human β -globin untranslated regions. SP64T derivatives containing the firefly luciferase reporter gene and the human CEA cDNA would thus be predicted to produce mRNA transcripts consisting of the luciferase and CEA ORFs, respectively, flanked by the β -globin untranslated regions. B, analyses of mRNA transcripts produced by *in vitro* synthesis using SP64T-derived plasmids. Luciferase and human CEA mRNA transcripts (1.0 µg) were size fractionated by agarose gel electrophoresis and visualized by UV illumination. Size markers indicate 1.9 and 2.5 kilobases (kb).

this analysis, it can be seen that the *in vitro* transcription reaction yielded CEA mRNA transcripts of approximately 2.5 kilobases. This size is in accord with the predicted transcript based on the CEA ORF plus β -globin untranslated regions and poly(A) sequences. Thus, these studies demonstrate that it is feasible to derive a homogenous population of mRNA species which corresponds to the human CEA coding region. Additionally, features have been incorporated into the transcript to enhance its expressivity in target cells.

To confirm the functional utility of the transcripts, analysis of their ability to accomplish human CEA expression *in vitro* was carried out. For these studies, mRNA transcripts were transfected

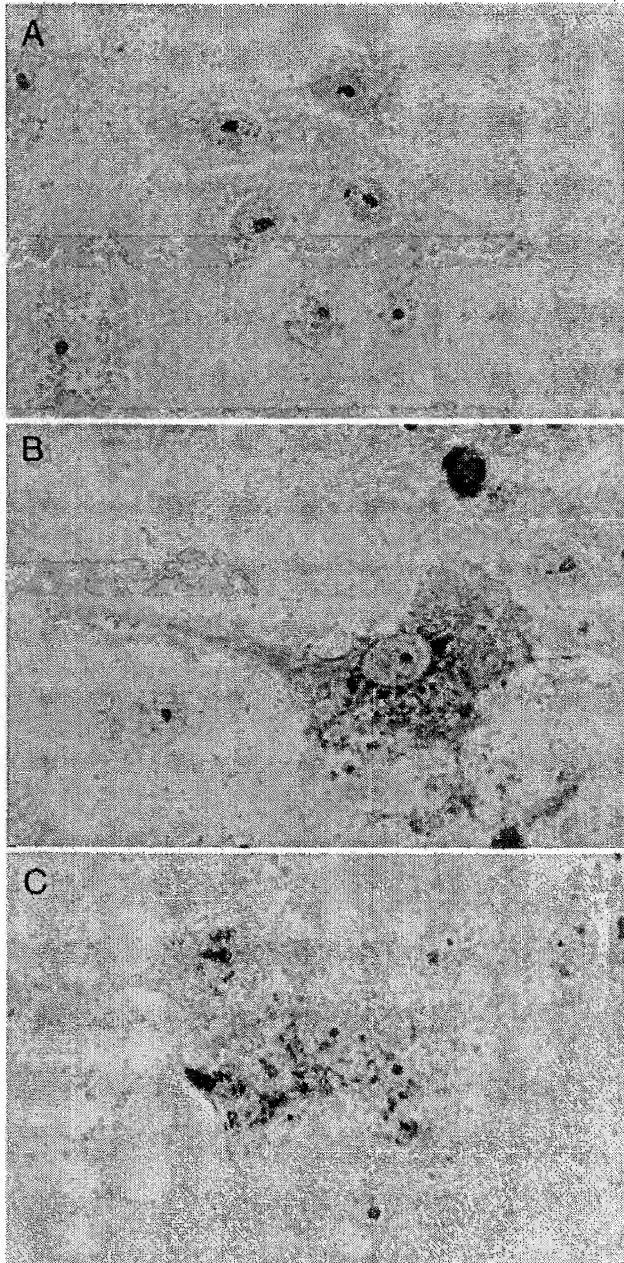


Fig. 2. Immunostaining for CEA in a mouse fibroblast cell line. No immunoreactivity was evident among control cells transduced with mRNA transcripts encoding firefly luciferase (A). CEA immunoreactivity was observed in cells transduced with CEA mRNA transcripts (B and C). Immunoreactivity was predominantly cytoplasmic with a perinuclear distribution (B). Original magnification $\times 600$.

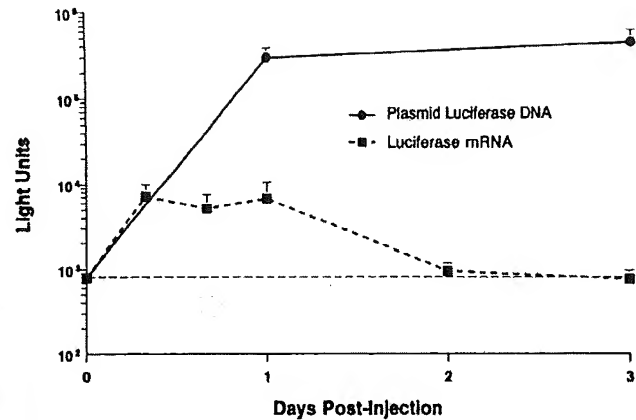


Fig. 3. *In vivo* expression of luciferase following i.m. injection of pGT28Luc mRNA or pCLuc4 DNA. Values indicate the mean light units \pm SEM for groups of five mice. A positive result is defined as exceeding 2 SD above the mean for 10 control muscles injected with saline (>700 light units) and is indicated by the dashed line. Luciferase activity from a 10- μ l aliquot of muscle extract was measured at various time points following a single 50- μ g injection of pGT28Luc mRNA or pCLuc4 DNA.

into target cells using a cationic liposome vector. In this regard, we have recently optimized this methodology and have shown that liposome-mRNA-mediated transduction can accomplish highly efficient gene transfer to a variety of target cells *in vitro* (19). We thus used these methods to transfect the murine fibroblast cell line WI62. This mesodermal cell line lacks endogenous CEA expression, minimizing the possibility of species cross-reactivity in this analysis. Cells were transfected as described in "Materials and Methods" and analyzed for CEA expression using immunohistochemistry and RIA techniques. By immunohistochemical analysis, cells transfected with luciferase control mRNA transcripts showed no evidence of CEA expression (Fig. 2A), whereas cells transfected with mRNA transcripts encoding human CEA demonstrated readily detectable human CEA expression (Fig. 2, B and C). To quantitate cell surface expression of CEA, RIA with ¹²⁵I-labeled COL-1 demonstrated 73,000 molecules bound/cell following transduction with CEA mRNA and 37,000 molecules bound/cell following transduction with plasmid DNA-encoding CEA. Transduction with the luciferase-encoding mRNA yielded 0 molecules bound/cell while the stably transfected CEA-expressing murine colon carcinoma line MC38-CEA-2 bound 108,000 molecules of COL-1/cell. Thus, it is feasible to produce mRNA transcripts encoding human CEA, and these transcripts are capable of directing expression of human CEA protein in heterologous target cells.

We next examined *in vivo* expression of plasmid DNA and mRNA transcripts in muscle using the luciferase reporter gene (Fig. 3). Intramuscular delivery of mRNA transcripts (pGT28Luc mRNA) produced luciferase activity which peaked 8 h postinjection at levels 10-fold above background and returned to baseline within 3 days. For comparison, i.m. delivery of plasmid DNA (pCLuc4 DNA) produced maximum luciferase activity 3 days postinjection at levels 630-fold above background. Thus, these mRNA transcripts encoding a firefly luciferase reporter are capable of directing readily detectable luciferase expression *in vivo* following injection into normal muscle.

The anti-CEA antibody response among mice immunologically primed with 50 μ g CEA mRNA twice weekly for 5 weeks followed by challenge with syngeneic, CEA-expressing colon carcinoma cells is provided in Table 1. Naive mice tumor challenged on the same day served as controls for the CEA-specific antibody response elicited by exposure to CEA-expressing tumor cells. Sera for antibody assays were obtained 3 weeks after tumor challenge. Five of seven mice primed

Table 1 Anti-CEA antibody response^a

Immunological priming	Mouse						
	1	2	3	4	5	6	7
Unprimed	0	6	0	0	0	0	0
CEA mRNA primed	0	101	70	0	72	95	94

^a Results expressed as ng ¹²⁵I-labeled CEA bound/ml sera (see "Materials and Methods"). A positive assay is defined as being >2 SD above the mean of normal mouse sera and is >20 ng/ml.

with CEA mRNA demonstrated CEA-specific antibody responses whereas zero of seven unprimed mice showed evidence of CEA-specific antibody response. Thus, immunization with mRNA transcripts encoding CEA can prime an immune response to CEA such that a CEA-specific antibody response is readily demonstrated following a booster immunization in the form of exposure to CEA-expressing tumor cells.

Discussion

Direct injection of naked plasmid DNA has been used to achieve antitumor immunization (8–10). For a variety of reasons, however, it would be advantageous to use an mRNA vector as an alternative to DNA vectors for this application. This study was designed to evaluate the feasibility of mRNA polynucleotide immunization. In this context, this study describes a method to generate mRNA transcripts which are capped, polyadenylated, and stabilized by human β -globin 5' and 3' untranslated regions. These constructs encode firefly luciferase or human carcinoembryonic antigen for *in vitro* and preliminary *in vivo* studies. The mRNA construct encoding human CEA directed CEA expression in mouse fibroblasts (WI62 cells) *in vitro* following liposome-mediated transfection. CEA expression was demonstrated by immunohistochemistry while cell surface display of the molecule was documented using cell surface binding of ¹²⁵I-labeled COL-1 (anti-CEA mAb) with intact viable cells.

The ability of such constructs to mediate protein expression *in vivo* was examined by i.m. injection of a single 50- μ g dose of mRNA transcripts encoding luciferase into the tongue muscle. Luciferase expression was readily demonstrated 8–24 h postinjection and returned to baseline within 3 days.

Given the ready demonstration of protein expression mediated by these constructs *in vitro* and *in vivo*, we examined the feasibility of using such a construct as a putative tumor vaccine. In this pilot experiment, mice received 50- μ g doses of mRNA transcripts encoding CEA twice weekly for 5 weeks by i.m. injection followed by challenge with 2×10^5 MC38-CEA-2 syngeneic tumor cells. Doses of 50 μ g were selected based on our previously reported optimization of polynucleotide immunization with plasmid DNA-encoding CEA using this tumor model (9). A schedule of injections twice per week was based on the observations by our group and others (18) that luciferase reporter gene expression following i.m. injection of mRNA transcripts peaks within 18 h and becomes undetectable 3 days postinjection. This dose and schedule primed an immune response to the tumor-associated antigen (CEA). The mRNA-immunized animals had readily measurable anti-CEA antibody 3 weeks after tumor challenge while the control animals had no evidence of antibody response following tumor challenge.

These studies support the feasibility of using mRNA polynucleotide vaccines to elicit an immune response. This strategy might be particularly useful to induce an immune response to a proto-oncogene product or growth factor which poses a risk of inducing malignant transformation or other adverse events mediated by prolonged protein expression in myofiber cells. Studies are under way to definitively examine the dose, schedule, immune response, and antitumor effects of CEA mRNA vaccination as we have previously reported for polynucleotide immunization with plasmid DNA (8–10). We have

plans to examine mRNA constructs encoding selected proto-oncogenes as well as strategies to amplify expression.

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